European Review for Medical and Pharmacological Sciences 2019; 23: 2596-2601

Effects of epidural infusion of morphine combined with small-dose naloxone on gastrointestinal interstitial cells of Cajal in rabbits

H. YANG[1,2], H. LUO[2], Y.-H. LI[1]

[1]Department of Anesthesiology, First Affiliated Hospital of Anhui Medical University, Anhui, China
[2]Department of Anesthesiology, Third Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China

Abstract. – OBJECTIVE: To study the effect of epidural infusion of morphine combined with small-dose naloxone on gastrointestinal interstitial cells of Cajal (ICC) in rabbits.

MATERIALS AND METHODS: A total of 80 healthy New Zealand rabbits were selected as objects of study, and divided into normal saline control group (Group NS, n=20), morphine group (Group M, n=20), naloxone group (Group N, n=20), and morphine + naloxone group (Group NM, n=20). Rabbits in four the groups received epidural catheterization for continuous drug infusion for 7 d, and epidural analgesia pump was connected. Visual analogue scale (VAS) score, intestinal propulsion rate, c-kit expression, and ICC count were detected and compared among four groups of rabbits.

RESULTS: No statistical differences of occurrence rates regarding constipation as well as expressions of c-kit and ICC count in the proximal colon were shown among rabbits in Group NS, Group N, and Group NM during drug administration ($p>0.05$). However, the occurrence rates of constipation of rabbits in Group M at 3-7 d were statistically higher than those in Group NS, Group N, and Group NM, and the differences were statistically significant ($p<0.05$). Moreover, the VAS scores in Group NS and Group N were significantly higher than those in Group M and Group NM, while the scores in Group M were also significantly increased compared to that in Group NM ($p<0.05$). The intestinal propulsion rates, expressions of c-kit and ICC counts of rabbits in Group NS, Group N, and Group NM were statistically higher than that in Group M ($p<0.05$).

CONCLUSIONS: Epidural infusions of morphine combined with small-dose naloxone effectively inhibit the gastrointestinal motility of rabbits via the reduction of ICC in the proximal colon of the gastrointestinal tract of rabbits. Moreover, small-dose of naloxone enhances the analgesic effect, and reduces the risk of adverse reactions.

Key Words:
Morphine, Small-dose naloxone, Epidural infusion, Gastrointestinal interstitial cells of Cajal in rabbits.

Introduction

Interstitial cells of Cajal (ICC) is a kind of gastrointestinal pacemaker cells, similar to human myocardial pacemaker cells, and abnormalities in its distribution and function may be one of the major causes of gastrointestinal motility disorders. ICC is, therefore, closely related to information transmission in smooth muscle cells and enteric nerve[1]. As a kind of long-acting and effective opioid analgesic, morphine is commonly used in clinical treatment. It selectively blocks the opioid receptors of pain transmission in the spinal cord, and activates the endogenous analgesic system. However, drawbacks concerning potent risk of adverse reactions exist and affect patients' satisfaction[2,3]. Naloxone, as a morphine receptor antagonist, is suitable for the diagnosis and treatment of opioid addiction and overdose poisoning. The combination use of naloxone with anesthesia in surgery relieves respiratory depression and exerts an arousal effect. Notably, it decreases the risk of adverse reactions caused by morphine, whereas barely influences the antagonistic effect of morphine[4]. However, the specific application dose of naloxone is still inconclusive in clinical practice[5]. A previous study[6] indicated the correlation between the induction of morphine on the colon slow transit motion of mice and the change in the proximal ICC. Based on this, we investigated the effect of epidural infusion of morphine combined with small-dose naloxone on gastrointestinal ICC in rabbits.

Corresponding Authors: Hong Luo, MD; email: hongluofg@163.com
Yuanhai Li, MD; email: yuanhaili65@163.com

Materials and Methods

Animal Materials

A total of 80 healthy New Zealand rabbits (either male or female) weighing 2.0-2.5 kg, provided by the hospital laboratory, were selected as objects of study, and divided into normal saline control group (Group NS, n=20), morphine group (Group M, n=20), naloxone group (Group N, n=20), and morphine + naloxone group (Group NM, n=20) using a random number table. All procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Methods

Rabbits in the four groups were intravenously injected with 4 mL/kg 20% urethane for anesthesia, and they were placed and effectively fixed on a test bench on the prone position under this condition. Epidural puncture was performed in L_{3-4} space of rabbits, and the catheter was implanted. After complete consciousness and recovery of motor and sense were observed, rabbits were anesthetized with 0.5 mL 1% lidocaine via epidural administration, suggesting successful epidural puncture and implantation. At 1 d after catheterization, CBI epidural analgesia pump was accurately connected. 0.8-1.0 μg/(kg·h) normal saline was infused into rabbits in Group NS. 9.2 μg/(kg·h) morphine was infused into rabbits in Group M. 0.92 μg/(kg·h) naloxone was infused into rabbits in Group N, and 9.2 μg/(kg·h) morphine. 0.92 μg/(kg·h) naloxone were infused into rabbits in Group NM. Rabbits in the four groups were infused continuously for 7 d. During infusion, the fixation and patency of catheter, as well as the infection, were closely examined and recorded. The catheter outlet and connector were regularly cleaned and disinfected, and the skin and epidural infections were prevented. At the same time, rabbits were routinely injected with the appropriate amount of penicillin sodium after operation for 3 consecutive days (twice per day), and the dressing was replaced in time.

Observational Indexes

1) *Pain degree.* Visual analog scale (VAS) was used to determine the pain in rabbits. After successful epidural puncture, the femoral vein was isolated via operation, and the cutting site of femoral vein was clamped using vessel forceps under the same force (upper 2 teeth) to produce an equal intensity of pain stimulation. The pain was evaluated using the VAS scoring method (a total of 0-10 points): 0 point (no pain and no movement of lower limbs), 1-3 points (mild movement of lower limbs), 4-5 points (moderate movement of lower limbs), and 6-10 points (violent movement of lower limbs). The pain value of rabbits was determined at 4:00 pm every day, and continuously observed and recorded for 7 d[7]. 2) *Occurrence rate of constipation.* The shape of faeces was recorded during drug administration using the following evaluation criteria: grade 1 (isolated hard mass), grade 2 (mass of faeces), grade 3 (cracked sausage-like faeces), grade 4 (soft sausage-like faeces), grade 5 (soft mass of faeces), grade 6 (muddy faeces), and grade 7 (watery faeces). The shape of faeces ≤ grade 3 was regarded as the criteria of constipation, and the occurrence rate of constipation was recorded accurately[8]. 3) *Intestinal propulsion rate.* At 1 d after withdrawal of morphine, rabbits were intravenously injected with 4 mL/kg 20% urethane. The gastric tube was inserted through the mouth under anesthesia, and the mixed solution prepared by 3 mL normal saline and 2 mL Chinese ink was injected. At 30 min after injection, 20 mL air was injected via the ear vein of rabbits to execute them. The abdomen was cut open, and the intestinal canal from pylorus to ileocecum was retained. The full length of the intestinal canal was measured in the absence of tension, and the propulsion distance of ink in the intestinal canal was measured. The intestinal propulsion rate was calculated: intestinal propulsion rate = propulsion distance of ink / full length of intestinal canal[9]. 4) *C-kit expression.* Proximal colon tissues and distal colon tissues (1 cm × 1 cm × 5 mm) were taken, embedded into paraffin routinely and sliced into sections. Sections were selected, followed by routine immunohistochemical staining of c-kit, and antigen retrieval via microwave. After that, SA1022-rabbit immunoglobulin G (IgG) antibody (diluted at 1:200) was added dropwise for incubation at 37°C overnight. Sections were washed with phosphate-buffered saline (PBS) for 3 times (2 min/time), and dropwise added with biotinylated goat anti-rabbit IgG antibody (diluted at 1:200), followed by washing with PBS, color development via diaminobenzidine (DAB), slight re-staining with hematoxylin, transparency with xylene, and sealing with neutral gum. The brown-stained cells were positive cells. Five fields of view (×200) were randomly selected on each section to count the number of positive cells, and the average amount was calculated. The gray scale was scanned using the

Table I. Comparison of occurrence rate of constipation among four groups of rabbits [n (%)].

Group	1 d	2 d	3 d	4 d	5 d	6 d	7 d
Group NS (n=20)	0 (0.0)[ab]	0 (0.0)[ab]	0 (0.0)[ac]	0 (0.0)[ac]	0 (0.0)[ac]	0 (0.0)[ac]	0 (0.0)[ac]
Group M (n=20)	1 (5.0)	3 (15.0)	17 (85.0)	19 (95.0)	20 (100.0)	20 (100.0)	20 (100.0)
Group N (n=20)	0 (0.0)[ab]	1 (5.0)[ab]	1 (5.0)[ac]	2 (10.0)[ac]	2 (10.0)[ac]	2 (10.0)[ac]	2 (10.0)[ac]
Group NM (n=20)	0 (0.0)[ab]	0 (0.0)[ab]	0 (0.0)[ac]	1 (5.0)[ac]	1 (5.0)[ac]	1 (5.0)[ac]	1 (5.0)[ac]

Note: In the pairwise comparison, [a]$p>0.05$. Compared with Group M, [b]$p>0.05$. Compared with Group M, [c]$p<0.05$.

RX image analysis system, and the gray value was calculated to present the expression level of c-kit[10]. 5) *ICC count.* After sections were selected, hematoxylin-eosin (HE) staining was performed, and pathological results were observed under an optical microscope (×400). Five ganglia were randomly selected on colonic myenteric plexus, and the number of ICC in each ganglion was counted. The average of ICC count in 5 ganglia was taken[11].

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (Armonk, NY, USA) statistical software was used for data analysis. Enumeration data were presented as percentage and case, and x^2-test was used for the intergroup comparison. Measurement data were presented as ($\bar{x}\pm s$), and *t*-test was used for the intergroup comparison. $p<0.05$ suggested that the difference was statistically significant.

Results

Occurrence Rate of Constipation

There were no statistically significant differences in the occurrence rate of constipation among rabbits in Group NS, Group N, and Group NM during drug administration ($p>0.05$). Likewise, the occurrence rates of constipation of rabbits in Group M at 1 and 2 d during drug administration showed no significant differences compared with those in Group NS, Group N, and Group NM ($p>0.05$). However, the occurrence rates of constipation of rabbits in Group M at 3-7 d were significantly higher than those in Group NS, Group N, and Group NM ($p<0.05$) (Table I).

Pain and Intestinal Propulsion Rate

The VAS scores of rabbits in Group NS and Group N were significantly higher than those in Group M and Group NM, while the scores were statistically higher in Group M than that in Group NM ($p<0.05$). In contrast, no significant differences of VAS scores of rabbits between Group NS and Group N were observed ($p>0.05$). The intestinal propulsion rates of rabbits in Group NS, Group N, and Group NM were significantly higher than that in Group M ($p<0.05$). There were no statistical differences in the intestinal propulsion rates of rabbits among Group NS, Group N, and Group NM ($p>0.05$) (Table II).

C-Kit Expression

The expressions of c-kit in the proximal colon of rabbits in Group NS, Group N, and Group NM were significantly higher than that in Group M ($p<0.05$). However, no statistical difference of the expression of c-kit in the proximal colon of rabbits was found among Group NS, Group N, and Group NM ($p>0.05$) (Table III).

ICC Count

Cell staining showed that brown-stained cells were positive cells (Figures 1, 2). The ICC counts in the proximal colon of rabbits in Group NS, Group N, and Group NM were significantly higher than that in Group M ($p<0.05$). But no significant difference of ICC count in the proximal colon of rabbits was detected among Group NS, Group N, and Group NM ($p>0.05$) (Table IV).

Table II. Comparisons of VAS score and intestinal propulsion rate among four groups of rabbits ($\bar{x}\pm s$).

Group	VAS score (points)	Intestinal propulsion rate(%)
Group NS (n=20)	9.3±0.6	67.3±13.0
Group M (n=20)	4.5±1.2[a]	50.4±13.2[a]
Group N (n=20)	9.1±0.8[bc]	66.0±12.3[bc]
Group NM (n=20)	3.4±1.1[acd]	65.3±13.5[bce]

Note: Compared with Group NS, [a]$p<0.05$, [b]$p>0.05$. Compared with Group M, [c]$p<0.05$. Compared with Group N, [d]$p<0.05$, [e]$p>0.05$

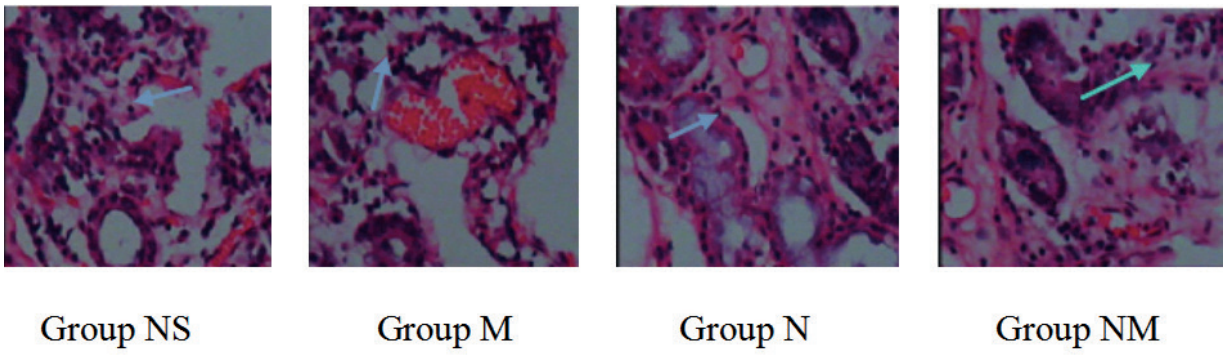

Figure 1. Staining images of proximal colon in four groups of rabbits (×400).

Discussion

Morphine and its derivatives are commonly-used drugs for relief and elimination of severe pain and exert a potent analgesic effect. Besides, they inhibit the smooth muscles in respiratory center, cough center, excitement bronchus, biliary tract, ureter, etc., and increase the tension of smooth muscles. Morphine can be distributed in the organs, such as the heart, stomach, liver, small intestine, and brain tissues. However, the action time of drug in each part varies with the administration route and dose[12]. Morphine, as a potent analgesic, has a certain effect of addiction. Long-term users depend strongly on morphine psychologically and physically. Thus, the limitations of its application needed to be strictly implemented with definite usage and dosage[13]. Studies have found that naloxone, as a morphine receptor antagonist, is suitable for the diagnosis and treatment of opioid addiction and overdose poisoning, and the small-dose naloxone has no antagonistic effect on the analgesic efficacy of morphine. Moreover, it can enhance the analgesic effect of morphine, and reduce the risk of nausea and vomiting, tolerance, respiratory depression, addiction, pruritus, and other related adverse reactions after application of morphine[14,15].

To avoid the influences of the side-effects, researchers proposed the epidural infusion of morphine and small-dose naloxone in clinical use[16]. It has been revealed that ICC in the myenteric plexus and the lower edge of circular muscle mucosa is a conductor and pacemaker of gastrointestinal motility, whereas tyrosine kinase receptor c-kit acts as a specific marker for ICC detection. C-kit receptor in digestive tract serves as a highly specific marker of the mastocyte. At the same time, the W site-associated mutant W/WV encoding mice with c-kit gene and the stem cell factor sl site-associated mutant sl/sld encoding its ligand show the loss of intestinal ICC and disappearance of intestinal slow wave. Therefore, by using c-kit immunohistochemical technique, ICC distribution in the gastrointestinal tract and abnormal distribution of ICC under pathological conditions can be specifically observed, thus providing a new way for the clinical medical research on a series of gastrointestinal motility disorders[17]. In this study, rabbits received a continuous epidural

Table III. Comparison of c-kit expression level among four groups of rabbits ($\bar{x}$±s).

Group	C-kit expression in the proximal colon	C-kit expression in the distal colon
Group NS (n=20)	3.9±2.5^{ab}	1.4±0.4^{b}
Group M (n=20)	2.2±1.4	1.5±0.2^{b}
Group N (n=20)	4.0±2.4^{ab}	1.5±0.4^{b}
Group NM (n=20)	3.8±1.7^{ab}	1.4±0.3^{b}

Note: Compared with Group M, $^{a}p<0.05$. In the pairwise comparison, $^{b}p>0.05$.

Table IV. Comparison of ICC count among four groups of rabbits ($\bar{x}$±s).

Group	ICC count in proximal colon	ICC count in distal colon
Group NS (n=20)	2.8±0.5^{ab}	1.0±0.5^{b}
Group M (n=20)	2.4±0.4	0.9±0.6^{b}
Group N (n=20)	2.8±0.4^{ab}	1.0±0.4^{b}
Group NM (n=20)	2.8±0.6^{ab}	1.0±0.4^{b}

Note: Compared with Group M, $^{a}p<0.05$. In the pairwise comparison, $^{b}p>0.05$.

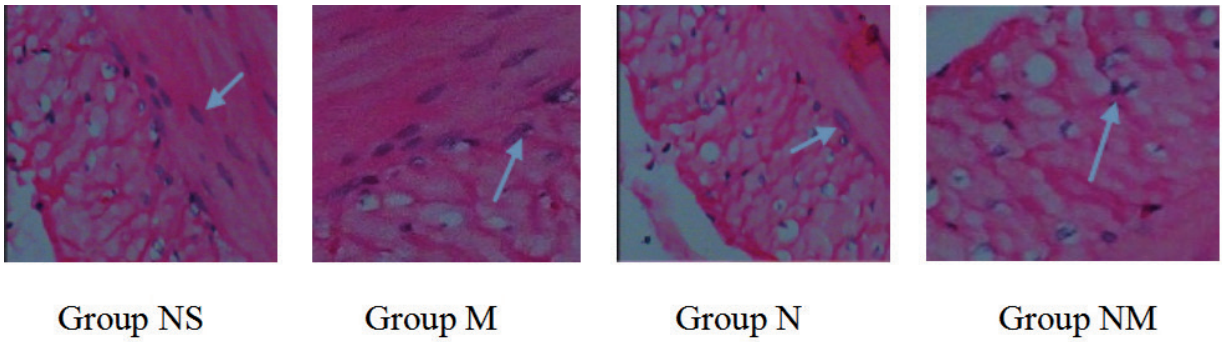

Figure 2. Staining images of distal colon in four groups of rabbits (×400).

infusion of morphine and small-dose naloxone for 7 d. Results showed that after the single infusion of morphine into rabbits, the occurrence rate of constipation showed an increasing trend over time, but there was basically no constipation after application of small-dose naloxone. The pain degree was significantly reduced, and both c-kit expression and ICC count in the proximal colon were basically restored[18]. The data indicated that the epidural infusion of morphine could inhibit the gastrointestinal motility of rabbits. The combined application of small-dose naloxone can enhance the analgesic effect of morphine, reduce the occurrence of adverse reactions, promote the gradual recovery of c-kit expression and ICC count in the proximal colon, making the gastrointestinal motility return to normal. Its mechanism of action may be related to the reduced ICC expression in the proximal colon, and exogenous naloxone reverses the change in ICC. A previous evidence[19] reported that intrathecal injection of morphine activated the N-methyl-D-aspartate receptor-caspase pathway and induced the apoptosis in the spinal dorsal horn of rats, suggesting that naloxone can reduce and reverse the morphine-induced apoptosis. Despite the development of novel pain-killer such as sufentanil in the application of postoperation[20], analgesic administration via the combination of morphine combined with small-dose naloxone could represent an opportunity to update the current practice, and clinical efficacy requires further evaluation within a large cohort of patients.

Conclusions

We demonstrated that epidural infusion of morphine into rabbits can inhibit the gastrointestinal motility function, and its mechanism is related to the downregulation of ICC expression in the proximal colon. Besides, combined epidural infusion of small-dose naloxone can reverse the morphine-induced ICC change, enhance the analgesic effect of morphine, and reduce the occurrence of adverse drug reactions, thereby improving the clinical acceptability.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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